COLORIMETRIC RESONANT REFLECTION AS A DIRECT BIOCHEMICAL ASSAY TECHNIQUE

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ABSTRACT

A novel approach for the detection of molecular interactions is presented in which a colorimetric resonant diffractive grating surface is used as a surface binding platform. A guided mode resonant phenomenon is used to produce an optical structure that, when illuminated with white light, is designed to reflect only a single wavelength. When molecules are attached to the surface, the reflected wavelength (color) is shifted due to the change of the optical path of light that is coupled into the grating. By linking receptor molecules to the grating surface, complementary binding molecules can be detected without the use of any kind of fluorescent probe or particle label. It is expected that this technology will be most useful in applications where large numbers of biomolecular interactions are measured in parallel, particularly when molecular labels will alter or inhibit the functionality of the molecules under study. High throughput screening of pharmaceutical compound libraries with protein targets, and microarray screening of protein-protein interactions for proteomics are examples of applications that require the sensitivity and throughput afforded by this approach.

INTRODUCTION

With the completion of the sequencing of the human genome, one of the next grand challenges of molecular biology will be to understand how the many protein targets encoded by DNA interact with other proteins, small molecule pharmaceutical candidates, and a large host of enzymes and inhibitors[1,2,3,4,5]. To this end, tools that have the ability to simultaneously quantify many different biomolecular interactions with high sensitivity will find application in pharmaceutical discovery, proteomics, and diagnostics. Further, for these tools to find widespread use, they must be simple to use, inexpensive to own and operate, and applicable to a wide range of analytes that can include peptides, small proteins, antibodies, and even entire cells.

Biosensors have been developed to detect a variety of biomolecular complexes including oligonucleotides, antibody-antigen interactions, hormone-receptor interactions, and enzyme-substrate interactions. In general, biosensors consist of two components: a highly specific recognition element and a transducer that converts the molecular recognition event into a quantifiable signal. Signal transduction has been accomplished by many methods, including fluorescence, interferometry[6,7], and gravimetry[8].

Of the optically-based transduction methods, direct methods that do not require labeling of analytes with fluorescent compounds are of interest due to the relative assay simplicity and ability to study the interaction of small molecules and proteins that are not readily labeled. Direct optical methods include surface plasmon resonance (SPR)[9], grating couplers[10], ellipsometry[11], evanascent wave devices[12], and reflectometry[13].

This paper describes a novel approach for the detection of molecular interactions in which a colorimetric resonant diffractive grating surface is used as a surface binding platform. The grating, when illuminated with white light, is designed to reflect only a single wavelength. When molecules are attached to the surface, the reflected wavelength (color) is shifted due to the change of the optical path of light that is coupled into the grating. By linking receptor molecules to the grating surface, complementary binding molecules can be detected without the use of any kind of fluorescent probe or particle label. The detection technique is capable of resolving changes of ~0.1 nm thickness of protein binding, and can be performed with the grating surface either immersed in fluid or dried. The readout system consists of a white light lamp that illuminates a small spot of the grating at nominally normal incidence through a fiber optic probe, and a spectrometer that collects the reflected light through a second fiber, also at normal incidence. Because no physical contact occurs between the excitation/readout system and the grating surface, no special coupling prisms are required and the grating can be easily adapted to any commonly used assay platform, such as microtiter plates and microarray slides. A single spectrometer reading may be performed in several milliseconds, thus it is possible to quickly measure a large number of molecular interactions taking place in parallel upon a grating surface, and to monitor reaction kinetics in real time.

Like ellipsometry, SPR, and reflectance spectrometry, the new method utilizes a change in the refractive index upon a surface to determine when a chemically bound material is present within a specific location. However, the method described in this paper incorporates a subwavelength structured surface (SWS) to create a sharp optical resonant reflection at a particular wavelength that can be tracked with high sensitivity as biological material is attached to the grating surface.

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Subwavelength structured surfaces are an unconventional type of diffractive optic that can mimic the effect of thin-film coatings. Previously described applications include their use as antireflection filters, polarizing filters, and narrowband filters[14,15]. An SWS structure contains a surface-relief grating in which the grating period is small compared to the wavelength of incident light so that no diffractive orders other than the reflected and transmitted zeroth orders are allowed to propagate. To design a SWS surface narrowband filter, a two-dimensional grating is sandwiched between a substrate and a cover layer that fills the grating grooves. When the effective index of refraction of the grating region is greater than the substrate or the cover, a waveguide is created. When the filter is designed properly, incident light passes into the waveguide region and propagates as a leaky mode. The grating structure selectively couples light at a narrow band of wavelengths into the waveguide. The light propagates only a very short distance (on the order of 10-100 micrometers), undergoes scattering, and couples with the forward- and backward-propagating zeroth-order light. This highly sensitive coupling condition can produce the resonant grating effect on the reflected radiation spectrum, resulting in a narrow band of reflected or transmitted wavelengths. The reflected or transmitted color of this structure can be modulated by the addition of biomolecular material to the upper surface of the structure. The added material increases the optical path length of incident radiation through the structure, and thus modifies the wavelength at which maxiumum reflectance will occur. A schematic diagram of a SWS structure is shown in Figure 1. The main requirements of the structure are that $n_2 > n_1$, that the layer thicknesses are selected to achieve resonant wavelength sensitivity to additional biomaterial on the top surface, and that the grating period is selected to achieve resonance at a desired wavelength. While the structures reported in this paper are fabricated from glass and silicon nitride dielectric materials, equivalent structures may be formed from embossed plastic with an appropriate dielectric cover layer.

An SWS structure may be used as a microarray platform by building a grating surface that is the same size as a standard microscope slide and placing microdroplets of high affinity chemical receptor reagents onto an x-y grid of locations on the grating surface. Likewise, an SWS structure can be built to be the same size as a standard microtiter plate, and incorporated into the bottom surface of the entire plate. When the chemically active microarray/microtiter plate is exposed to an analyte, molecules will be preferentially attracted to locations that have high affinity. Some locations will gather additional material onto their surface, while other locations will not. By measuring the shift in resonant wavelength within each individual microarray/microtiter plate location, it is possible to determine which locations have attracted additional material. The extent of the shift can be used to determine the amount of bound analyte in the sample and the chemical affinity between receptor reagents and the analyte.

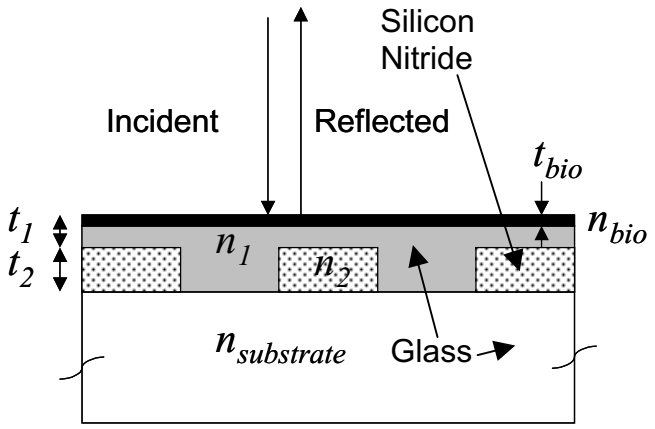

Figure 1.
Schematic diagram of the optical grating structure used for the colorimetric resonant reflectance biosensor.

MATERIALS AND METHODS

Sensor Fabrication

Biosensor fabrication begins with a flat glass substrate (Corning) that is coated with a thin layer (180 nm) of silicon nitride by plasma-enhanced chemical vapor deposition (PECVD). Because the desired grating structure requires holes and spaces with a period less than 1 μm over a large area, conventional photolithographic methods such as UV exposure through a photomask or direct writing of photoresist with an electron beam are not accurate or efficient methods. Instead, the desired structure is first produced in photoresist by coherently exposing a thin photoresist film to three laser beams, as described in previous research[16]. The nonlinear etching characteristics of photoresist are used to develop the exposed film to create a pattern of holes within a hexagonal grid. The photoresist pattern is transferred into the silicon nitride layer using reactive ion etching (RIE). The photoresist is removed, and a cover layer of spin-on-glass (SOG) is applied (Honeywell Electronic Materials) to fill in the open regions of the silicon nitride grating.

Sensor Readout Instrumentation

In order to detect the reflected resonance, a white light source illuminates a ~1 mm diameter region of the grating surface through a 100 micrometer diameter fiber optic and a collimating lens. Smaller or larger areas may be sampled through the use of illumination apertures and different lenses. A detection fiber is bundled with the illumination fiber for gathering reflected light for analysis with a spectrometer (Ocean Optics). The currently used spectrometer is centered at a wavelength of 800 nm, with a resolution of ~0.14 nm

between sampling bins. The spectrometer integrates reflected signal for 5-75 msec for each measurement. The biosensor sits upon an x-y motion stage so that different regions of the biosensor surface can be addressed in sequence.

Equivalent measurements may be made by either illuminating the top surface of device, or by illuminating through the bottom surface of the transparent substrate. Illumination through the back is preferred when the sensor active surface is immersed in liquid, and is most compatible with measurement of the sensor when it is incorporated into the bottom surface of a microwell plate.

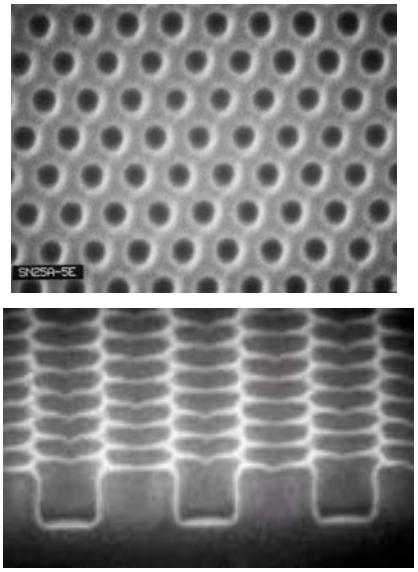

Figure 2.
SEM photographs of photoresist grating structure in plan view (top) and cross section (bottom).

Surface Activation Chemistry

Before the sensor is activated for the immobilization of receptor molecules, it was first cleaned in piranha solution (70/30 % (v/v) concentrated sulfuric acid/30% hydrogen peroxide) for 12 hours and then washed thoroughly with deionized water. To activate the surface with amine functional groups that can be used as a general-purpose surface for subsequent covalent binding of linker molecules, a piranha-cleaned sensor was immersed in 3% of 3-aminopropyltriethoxysilane (Pierce) in dry acetone (Sigma) for 1 minute, which was then rinse with dry acetone, dried in air, and washed in water.

After activating the sensor with amine, a bifunctional linker molecule can be attached to the sensor. The linker molecule, in turn, binds the receptor molecule that is used for specific recognition of a biochemical analyte. To activate an amine-coated sensor with biotin, 2 ml of NHS-PEG-biotin (Shearwater) solution in TPBS (pH 8) at 1.0 mg/ml concentration was added to the sensor surface, and incubated at 37°C for 1 hour. The sensor was then washed with TPBS.

After biotin has been immobilized on the sensor surface, the sensor bound readily to either goat anti-biotin IgG or streptavidin. All reagents used in the biosensor, including goat anti-biotin IgG, anti-goat IgG, pepsin, steprtavidin (SA)-cy3, and bovine serum albumin (BSA), were purchased from Sigma and used as packaged.

RESULTS

Measurement of Resonant Reflection

Figure 3 shows the resonant reflectance spectra taken from the fabricated structure using the instrumentation described above. The reflection efficiency was measured by referencing the sensor reflectance with than of an aluminum mirror.

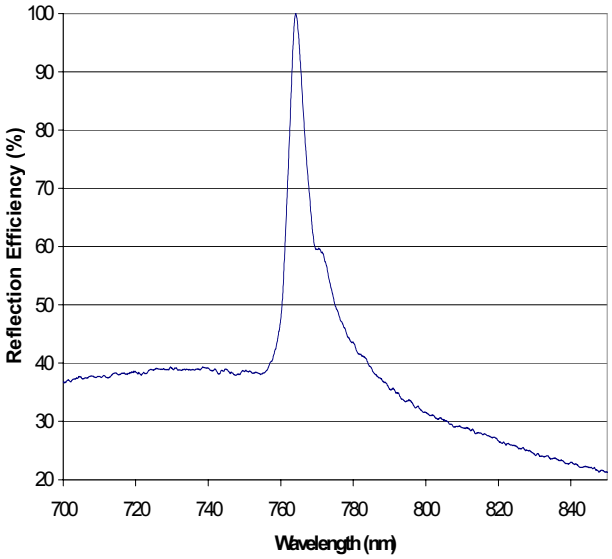

Figure 3.
Measured reflectance spectra of the biosensor demonstrating 100% reflection efficiency referenced to an aluminum mirror at the resonant wavelength.

Protein-Protein Binding Assay

To demonstrate the application of the biosensor for protein binding assays, the grating surface, prepared with a biotin surface using the described protocol, was used to detect goat anti-biotin antibody (Pierce). Figure 4 shows the results of an experiment in which a single sensor location is measured continuously through the course of consecutively adding various molecules to the surface. Throughout the experiment, the detection probe illuminates the sensor through the back of the sensor substrate, while biochemistry is performed on the top surface of the device. A rubber gasket perfusion chamber was sealed around the measured sensor location so that added reagents would be confined, and all measurements were performed while the top surface

of the sensor was immersed in buffer solution. After initial cleaning, the sensor was activated with NH_2, and an NHS-Biotin linker molecule. Goat anti-biotin IgG of several different concentrations (1, 10, 100, 1000 µg/ml; volume = 100 µl) were consecutively added to the sensor and allowed to incubate for 30 minutes while the peak resonant wavelength was monitored. Following application of the highest concentration anti-biotin IgG, a second layer of protein was bound to the sensor surface through the addition of anti-goat IgG at several concentrations (0.1, 1, 10, and 100 µg/ml; volume = 100 µl). Again, the resonant peak was continuously monitored as each solution was allowed to incubate on the sensor for 30 minutes.

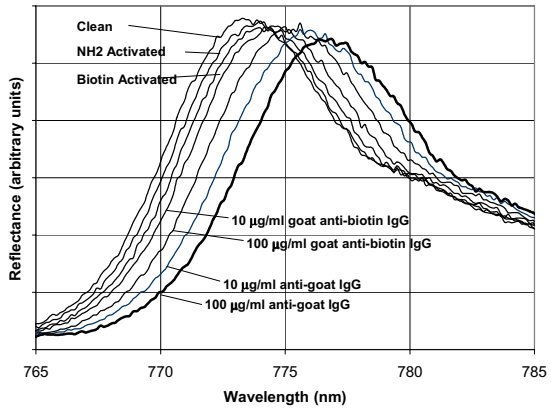

Figure 4.
Measured shifting of the resonant wavelength caused by the binding of various biomolecular layers.

Detection Sensitivity

The peak wavelength shift induced by dried protein on the sensor surface was measured to determine detection sensitivity separately from the effectiveness of receptor binding chemistry. Bovine serum albumin (BSA) was dissolved in water at various concentrations, and applied to the sensor by application of a 3µl droplet. The droplets were allowed to dry in air, leaving a small quantity of BSA distributed over a ~2 mm diameter area. The illumination optics of the readout instrument probes a ~1 mm diameter spot within the center of the dried protein area. The PWV of the biosensor was measured before and after droplet deposition, and the PWV shift was recorded. As shown in Figure 5, a wide range of BSA concentration was studied – from 0.001 µg/ml to 10,000 µg/ml. At concentrations above 10,000 µg/ml, the dried solution formed an opaque layer that saturated the sensor response. An approximately logarithmic dependence between peak wavelength shift and protein solution concentration was measured, with a minimum detectable concentration of 0.001 µg/ml. After drying of the carrier liquid, this concentration corresponds to a density of 4.2×10^{-13} g/mm^2 of BSA deposited over the dried spot area.

Finally, a series of assays were performed to detect streptavidin binding by a biotin receptor layer. A sensor was first activated with the amine protocol, followed by attachment of a NHS-PEG-Biotin linker layer, as previously described. Next, 3 µl droplets of streptavidin in PBS were applied to the sensor at various concentrations. The droplets were allowed to incubate on the sensor surface for 30 min before thorough washing with PBS and DI water. The PWV was measured before and after streptavidin binding, and the PWV shifts recorded between the two measurements are shown in Figure 6. A linear relationship between PWV shift and streptavidin concentration was observed, and the lowest streptavidin concentration measured was ~0.1 – 1.0 ng/ml. The negative control using exposure to only PBS solution did not register a peak wavelength shift. A 1.0 ng/ml concentration of streptavidin corresponds to a molarity of 0.0167 nM.

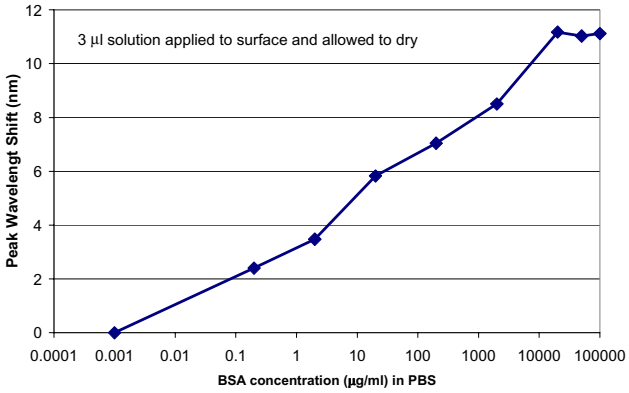

Figure 5.
PWV shift measured on the surface of a sensor upon which 3 µl droplets of BSA solutions of various concentration were deposited and allowed to dry.

CONCLUSION

A novel approach for the detection of molecular interactions was described in which a colorimetric resonant diffractive grating surface is used as a surface binding platform. The sensor operates by reflecting an extremely narrow band of wavelengths when illuminated with collimated white light, and detecting shifts in the reflected wavelength when biomolecular material is added or removed from the surface. The sensor can be used as a general-purpose platform for biochemical assays by linking receptor molecules to the grating surface and detecting complementary binding molecules in a test sample without the use of any kind of fluorescent probe or particle label.

Several types of assays were performed to demonstrate the use of this phenomenon as a biosensor, and to explore its sensitivity resolution limits. Preliminary results indicate that

detection sensitivities of 0.016 nM are readily obtained, and several aspects of the sensor design, readout system design, and surface chemistry efficiency have yet to be fully optimized.

It is expected that this technology will be most useful in applications where large numbers of biomolecular interactions are measured in parallel, particularly when molecular labels will alter or inhibit the functionality of the molecules under study. High throughput screening of pharmaceutical compound libraries with protein targets, and microarray screening of protein-protein interactions for proteomics are examples of applications that require the sensitivity and throughput afforded by this approach. In future work, an equivalent sensor structure will built in large areas using a plastic embossing process that can be inexpensively incorporated into common disposable laboratory assay platforms such as microtiter plates and microarray slides. Future work will also present more detailed experiments to quantify detection sensitivity, particularly with respect to the detection of small molecule interactions.

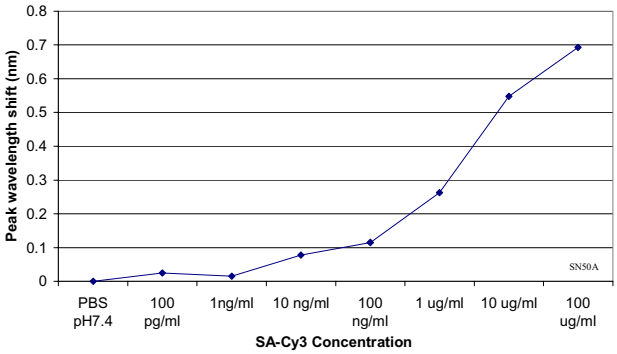

Figure 6.
PWV shift as a function of streptavidin concentration for a biotin-activated biosensor. The sensor was exposed to 3 µl droplets of streptavidin solution that were allowed to incubate for 30 minutes before rinsing and drying.

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